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Differential expression of Toll-like receptor genes (TLR2 and TLR4) across different tissues of riverine buffalo

P K DUBEY[1], S GOYAL[2], NAMITA[3], S K MISHRA[4], S K GAHLAWAT[5] and R S KATARIA[6]

National Bureau of Animal Genetic Resources, Karnal, Haryana 132 001 India

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ABSTRACT

Toll-like receptors (TLRs) are one of the important pattern recognizing receptors which are responsible for the induction of innate as well as adaptive immune response against a wide range of microbial components. Among different TLRs, TLR2 and TLR4 are expressed on the cell surface identifying Gram-positive and Gram-negative bacterial components. The present study was undertaken to analyze the expression of TLR2 and TLR4 genes in different buffalo tissues by using highly sensitive real-time PCR technique. Although the expression of both the genes was observed in all the 8 different tissues taken for the present study, the maximum expression of TLR2 was seen in blood followed by skin, lungs and spleen and the lowest expression was found in the uterus. TLR4 showed maximum expression in blood followed by skin, lungs, mammary gland of non-lactating, lactating buffalo and heifer and the lowest expression was seen again in the uterus.The findings suggest both the genes having important functions in blood and skin, the first line of protection for pathogenic challenge apart from other organs in buffalo.

Key words: Buffalo, Expression profile, Real-time PCR, Toll-like receptors

Toll-like receptors (TLRs) is a family of pattern recognition receptors, mainly expressed on the surface of immune cells against the variety of pathogen-associated molecular patterns (PAMPs) of various pathogens (Roach *et al.* 2005) and enhance the signal transduction for their immunogenic clearance. To date, 13 members of the TLR family have been identified in mammals and 10 in livestock species (Dubey *et al.* 2012). Expression of TLRs varies in different range of organs and tissues (Iqbal *et al.* 2005) and the variations in the expression of different TLRs are associated with the immune responsiveness and disease progression (Helmby and Grancis 2003).

TLR2 and TLR4 recognize Gram-positive and Gram-negative bacteria respectively, to mediate host response and variations in these TLRs are linked with incidences of various diseases, such as tuberculosis in human (Xiong *et al.* 2012), mastitis (Sentitula *et al.* 2011) etc. So, to understand the mechanism behind the generation of immune response by different cells and tissues, the TLRs mRNA abundance, as indicator of gene expression, needs to be determined. In previous reports, several methods were reported for the expression analysis of specific genes, but still little information is available on the TLRs gene expression by the real-time PCR assay. The tissue distribution of the mRNA expression was previously reported in various species by using the semi-quantitative PCR e.g. in mice (Prueet *et al.* 2004), chickens (Iqbal *et al.* 2005), bovines and ovines (Menzies and Ingham 2006), buffalo (Vahanan *et al.* 2008) and goat (Thirumurugan *et al.* 2009), but highly sensitive technique of real time PCR has not been used yet. The buffalo is a very important livestock species, playing significant role in Indian economy by contributing to dairy as well as meat industry, but its distribution being limited to southeast Asia, its genetic potential has not been fully exploited. This is the first ever report on the documentation of tissue distribution of different bacterial ligand recognizing TLR2 and TLR4 genes of buffalo by using the real-time PCR assay across the different tissues.

MATERIALS AND METHODS

Eight different tissues (spleen, mammary-gland (lactating), mammary-gland (heifer), mammary gland (non-lactating), lung, skin, blood, uterus) of 3 healthy buffaloes were collected from Delhi Slaughter House, Gazipur and were kept in RNALater (Life Technologies) till brought to lab. The RNALater was decanted completely and the tissues were stored at –80°C, until further used for RNA isolation.

RNA isolation and cDNA synthesis: Total RNA was isolated from approximately 100 mg of homogenized tissue,

Present address: [1](prvn.dubey@gmail.com), WPI-IFREC, Osaka University, Osaka, Japan. [2](shubhamgoyal28@gmail.com), RIKEN Centre for Life Sciences, Yokohama, Japan. [3,4]Research Scholar (drnamitavet@gmail.com, btmayank09@gmail.com), [6]Principal Scientist and Head (katariaranji@yahoo.co.in), Animal Biotechnology Division. [5]Head (skgcdlu@gmail.com), Department of Biotechnology, Chaudhary Devi Lal University, Sirsa.

Table 1. Real time primer sequence and other details utilized for expression analysis of TLR2, TLR4 and housekeeping RPS15 genes of buffalo

Gene	Primer sequence (5'-3')	Annealing Temp. (°C)	Size (bp)	Ligand
TLR2	For. ACCTGCCGTCTGATTTTCTTTCTG Rev. GGTGGCGGTCTTCGTTTCAA	60	119	Gram-Positive bacteria
TLR4	For. CCCTTTCAGCTCTGCCTTCACTAC Rev. CAGGTCTGGGCAATCTCATACTCG	56	143	Gram-Negative bacteria
RPS15	For. CAGCTTATGAGCAAGGTCGT Rev. GCTCATCAGCAGATAGCGCTT	56	130	Internal control gene; Structural constituent of ribosomes

by TRIzol method (Invitrogen) and further purified and treated with DNaseI using RNeasy kit as per manufacturer's instructions. Quality and quantity of purified RNA was assessed by UV spectrophotometer. cDNA was prepared by using oligo (dT)18 primers from 2 µg of the total RNA of each sample using cDNA synthesis kit, following manufacturer's protocol. Real time primers were designed from buffalo TLR2 (Acc no. HM756162) and TLR4 (Acc no. HQ343416) along with housekeeping gene RPS15, designed from the reported cattle sequence (Acc no. NM_001192201.1) using PrimerSelect program of Lasergene software and were synthesized from Integrated DNA Technologies (Table 1).

Expression profiling of bacterial TLR2 and TLR4 genes and data analysis: Quantitative real-time PCR was performed in 96-well real-time PCR system using SYBR Green 1 according to manufacturer's instructions. Standard curve for the housekeeping gene RPS15 was drawn on 10-fold dilutions of cDNA having efficiency in the range of 96–107% and slope in the range from –3.1 to –3.6. Technical replicates (2) and biological replicates (3) were used for each sample. Gene expression values were then calculated for each TLR using "Ct method, mean threshold Ct values were determined and the mean results were expressed in the form of mean normalized expression, subtracting the values of housekeeping gene. The fold change was calculated by taking one of the tissues as control by $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001).

RESULTS AND DISCUSSION

Toll-like receptors hold the ability to differentiate self from non-self and are not only important in immune response but are also associated with many autoimmune and inflammatory diseases like asthma, arthritis, allergies, atopic dermatitis, atherosclerosis, Crohn's disease and even cancer in human beings (Nicolas *et al.* 2005). This vital process of generation of inflammatory responses basically depends upon the location and abundance of each TLR potentially involved in this process (Cario *et al.* 2002) and due to this reason it becomes essential to establish the expression profiling of these biologically diverse molecules in various tissues. Since in livestock species, such role of TLRs is yet to be established, keeping in mind that point, the distribution of bacterial ligand recognizing TLRs was analysed in 8 different tissues of buffalo by using the most accurate and highly sensitive method of real-time PCR assay.

Buffalo TLR2 was expressed in almost all the tissues included in the present study (Table 2). But the maximum expression of TLR2 was seen in blood, skin, lungs and spleen and the lowest expression was found in lactating, non-lactating, heifer mammary and uterus tissues of buffalo. Further, fold change was calculated by taking uterus as the control. Many times multiplicative expression was seen in blood, skin and spleen (Fig. 1a). In earlier reports, by semi-quantitative RT-PCR, it was reported that tissues such as the spleen, liver, ovary and lung, showed consistently higher levels of TLR2 in buffalo (Vahanan *et al.* 2008) and the lowest in uterus. Menzies and Ingham (2006) reported the highest expression of TLR2 in bovine skin as compared to other epithelial cells, which implies the effective capability of this tissue to respond to the Gram-positive bacterial infections. But some different types of results were reported in goats, where the maximum expression of TLR2 was seen

Table 2. Average Ct, ΔCt and fold change values of bacterial TLR2 and TLR4

Tissue	Av. Ct values			ΔCt		Fold change*	
	TLR2	TLR4	RPS15	TLR2	TLR4	TLR2	TLR4
Spleen	25.29	26.25	22.18	3.11	4.07	9.713	39.39
Mammary gland (non-Lac)	27.02	24.52	20.18	6.84	4.34	1.366	32.67
Mammary gland (Lac)	25.72	24.91	20.29	5.43	4.62	1.94	26.90
Mammary gland (heifer)	25.25	25.8	19.82	5.43	5.98	1.94	10.48
Lung	24.58	24.8	20.17	4.41	4.63	3.94	26.72
Blood	21.75	22.56	19.9	1.85	2.66	23.26	104.69
Skin	24.05	24.34	22.1	1.95	2.24	21.70	140.06
Uterus	29.34	32.32	22.95	6.39	9.37	-	-

* Uterus taken as control to calculate fold change.

in PBMC and the lowest in skin (Tirumurugan *et al.* 2010).

Buffalo TLR4 was also found to be expressed in all the tissues taken for the present study (Table 2), indicating the ability of all these cells and tissues to identify PAMPs of Gram-negative bacteria, an indication of their higher adaptability to fight against bacterial infections. The highest expression of TLR4 was in blood followed by skin, lungs, mammary gland of non-lactating, lactating buffalo and heifer and the lowest expression was seen in uterus and spleen. In TLR4, when the fold change among the tissues was calculated by taking uterus as control, multiplicative fold change was seen in blood and skin with other tissues having somewhat lower fold change (Fig. 1b). The similar results of mRNA expression of TLR4 have been reported in buffalo by semi-quantitative method. There also the highest expression of TLR4 was found in blood and the lowest was found in spleen and uterus (Vahanan *et al.* 2008). In human also, the maximum expression of TLR4 has been observed in spleen and lowest in liver (Rock *et al.* 1998). In murines, the maximum expression of TLR4 was seen in lung, heart, spleen, kidney and liver (Qureshi *et al.* 1999), indicating that the basal tissue expression might not be identical across tissues and species, despite the homologous promoter region. In bovine, lowest expression of TLR4 was studied in skin and ovine gut in comparison to other tissues and cells (Menzies and Ingham 2006). Very low expression of TLR4 in goat was reported in jejunum, uterus and highest level in PBMC and secondary lymphoid organs (Tirumurugan *et al.* 2010).

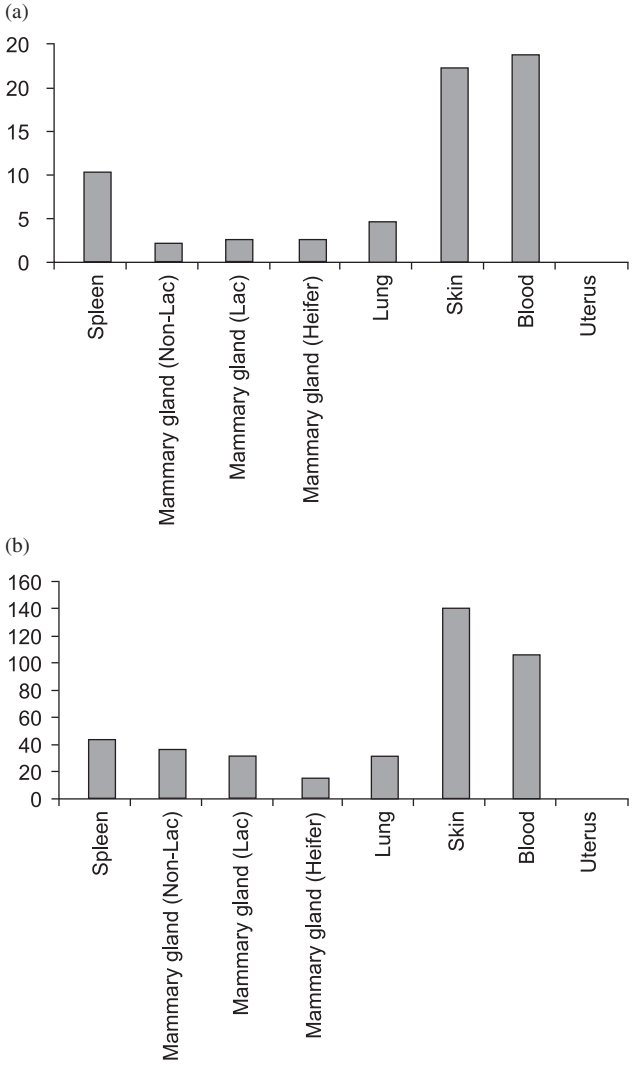

Fig. 1. Fold change taking uterus as control (a) buffalo TLR2 and (b) TLR4 genes across different buffalo tissues.

TLRs expression within tissue and individual's capability to respond against the microbial challenge is governed by the entry of pathogens in the target organ of host's body. Although the importance of an organ or tissue cannot be confirmed by the mere presence of TLRs only, as different tissues consist of different cell types. A minimum number of specific cell types, which may be important in immune recognition although existing in the organ, but their pattern recognition receptors may not be detected by the whole organ analysis Nevertheless, such studies have helped in documentation of target TLRs expression in different tissues both at protein and mRNA levels (Sebastiani *et al.* 2000).

This study thus showed the variable distribution of TLR2 and TLR4 genes in different buffalo tissues using highly sensitive real-time PCR technique, for the first time. This information will be important in assessing the feasibility of measuring the mRNA expression levels of target genes in selected tissues and cells and which can be further used in studying their role in generation of different immune responses against the different microbial ligands in buffalo, an important Indian livestock species.

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